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Appendices

Appendix A: Detailed methods for animal maintenance, trematode enumeration, leukocyte and kidney volume quantification, and statistical analyses.
***Ecological Archives* A/E/M000-000-A#.**

Appendix B: Statistical results for effects of a caged predator, tadpole density and trematode exposure on tadpole development, survival and activity.
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Appendix C: Graphs showing effects of density and delayed tadpole development on susceptibility to *Echinostoma trivolvis* infection.
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Appendix D: Statistics and graphs describing effects of density, predation and development on leukocyte parameters.
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Appendix A. Detailed methods for animal maintenance, trematode enumeration, leukocyte and kidney volume quantification, and statistical analyses.

Animal collection and maintenance

Twenty toad (*Bufo americanus*) egg masses were collected from a pond in central PA (N 40° 45' 51.4", W 78° 0' 58.6") on April 30, 2007, and tadpoles were raised in outdoor kiddie pools covered with 25% shade-cloth and fed rabbit chow ad libitum until the start of experiment. At the start of both experiments, tadpoles were Gosner stages 26-28 and had an average mass of 25.3 mg. Planorbid snails (*Planobella trivolvis*) were collected from a semi-permanent pond in northwestern PA (N 41° 35' 17.8", W 80° 14' 41.7"), maintained in shaded outdoor wading pools in well water supplemented with crushed chalk, and fed rabbit chow and boiled lettuce ad libitum to supplement natural algal growth. Snails were screened for trematode infections by placing individuals in 75 mL of artificial spring water (ASW; Cohen *et al.* 1980) overnight and checking for the presence of cercariae with a dissecting microscope. Adult newts (*Notophthalmus viridescens*) were collected from a permanent pond in central PA (N 40° 39' 12.2", W 77° 54' 9.6"). Animal maintenance and experiments were conducted at the Penn State Agricultural Research farm at Rock Springs, PA.

During experiments, tadpoles were held in 30-L plastic tubs. These mesocosms were filled with 19 L of well water on June 6, inoculated with zooplankton from local ponds and 1 g rabbit chow to supplement natural algal growth, and covered with 25% shade-cloth. Tadpoles were added on June 11. Water was changed once per week and new rabbit chow added after each water change. Mesocosm temperatures were approximately 24-29°C.

Trematode enumeration and identification

Toad specimens were stained and cleared for trematode enumeration using a modification of the Hanken and Wassersug protocol (1981). Intestines were removed prior to staining and specimens were transferred to 70% ethanol (30% water) overnight, followed by one change of ethanol and transfer into 1% hydrogen peroxide in 70% ethanol. Additional hydrogen peroxide was added daily until specimens were bleached (no visible melanin). Specimens were then stained overnight with 50% Semichon's acetic carmine, rinsed twice in 70% ethanol, and decolorized in 70% acid ethanol (1% hydrochloric acid) for several hours until tissues were pale pink. Decolorization was halted by replacing half the acid ethanol with basic ethanol (1% sodium hydroxide) and specimens were transferred to 100% glycerol to render tissues transparent. Cysts were counted in the kidneys (pronephros and mesonephros) using a compound microscope. Trematode identity was confirmed as described by Raffel *et al.* (in press), by completing the life cycle in Golden hamsters (*Mesocricetus auratus*) to obtain adult specimens. An adult voucher specimen of *E. trivolvis* was deposited in the U.S. National Parasite Collection (USNPC 101920).

Leukocyte profiles and kidney volumes

Tadpoles were euthanized by decapitation and blood was collected with a heparinized capillary tube. Blood was then applied to a microscope slide as a thick smear. Smears were air dried for 10 minutes, fixed in methanol, and stained using the benzidine-Giemsa procedure of Raffel *et al.* (2006).

Blood smears were examined at 400x magnification counting all leukocytes (lymphocytes, neutrophils, eosinophils, basophils, monocytes, and thrombocytes) in non-overlapping fields of view (4-29 fields of view examined depending on size of smear). The observer was blind to treatment assignments. To calculate the abundance of each cell type relative to erythrocytes, erythrocytes were counted in 10 randomly selected

fields of view within the smear. Fields of view with excessive cell lysis were passed over, and slides with fewer than 50 counted leukocytes were excluded from the analysis. This yielded 110 usable blood smears, all but 8 with >100 leukocytes counted.

To measure kidney volume in each of these tadpoles, a digital photograph was taken of the left pronephros at 320x magnification, and the area of the pronephros (in mm^2) was measured three times using the program ImageJ (Rasband 1997-2009). Because the pronephros is roughly spherical, its volume was estimated using the formula $4/(3\pi^{1/2})*Area^{3/2}$.

Detailed statistical methods

The mesocosm was the unit of replication in all analyses, so summary statistics were calculated for each mesocosm (i.e., arc-sine square root transformed proportion dead, mean time to metamorphosis, mean mass at metamorphosis, mean activity level, and mean echinostome intensity), and ANOVA was used to test for interactive effects of density, a caged predator and trematode exposure. Spatial block and treatment interactions were included in all models regardless of significance, except when testing for a relationship between two uncontrolled biological variables (e.g., Gosner stage and leukocytes). We used survival regression with a gaussian error distribution to estimate mean time to metamorphosis for each tank, censoring individuals that died or failed to metamorphose before the end of the experiment (R function "survreg" in package "survival"). Mean mass at metamorphosis only included individuals that reached metamorphic climax by the end of the experiment (removed when tail < 1 cm long). The proportion of tadpoles moving per observation was arcsine-square-root transformed before calculating mean activity for each mesocosm.

For the Exposure experiment, predator and density effects on the mean log-transformed trematode burden (log[burden + 1]) were assessed with ANOVA. The expectation was that mean trematode burdens in the high density treatment would be approximately half that in the low-density treatment, since there were twice as many tadpoles to be infected by the same number of cercariae. We therefore also estimated the total number of encysted trematodes per mesocosm (total trematodes) by multiplying mean trematode burden per individual by the number of tadpoles in the mesocosm (15 or 30). Exposure duration for a mesocosm was estimated using the time to metamorphosis of individuals whose cysts were counted. Total trematodes fit a negative binomial distribution, so we used a negative binomial generalized linear model (glm) with a log link to test for treatment effects. To model the effect of exposure duration on total trematodes, we again used a negative binomial glm. However, we constrained the intercept to pass through the origin because tadpoles had zero infections at the start of the experiment, and we used an identity link because a constant average rate of cercarial shedding should cause trematode burden to increase linearly through time (instead of log-linearly). We conducted an ANOVA on the residuals from the exposure duration model to test for the effect of tadpole density and predation on total trematodes independent of among-tank variation in exposure duration. It would have been inappropriate to test for these effects by including exposure duration as a covariate in a multiple regression, because it is invalid to exclude the intercept from a model with categorical variables. All analyses of trematode burden and total trematodes were weighted by the number of individuals examined per tank.

For the Susceptibility experiment, individual encystment rates (proportion encysted out of 30 cercariae) were arcsine-square-root transformed before calculating a mean for each mesocosm and testing for effects of predation and density using ANOVA. Models were weighted by the sample size available from each mesocosm. Because density influenced tadpole development and kidney volume, both of which could plausibly explain density effects on susceptibility to echinostome infection, we used variance partitioning to estimate what proportion of the density effect on trematode encystment could be explained by variation in tadpole development or kidney volume (Gotelli and Ellison 2004). Interactive effects of predation and density on mean leukocyte counts (neutrophils, lymphocytes, eosinophils, basophils, monocytes) were assessed using MANOVA, weighted by the number of usable blood smears in a mesocosm. Univariate effects on individual leukocyte types and on the neutrophil-to-lymphocyte ratio were tested using ANOVA.

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Appendix B. Statistical results for effects of a caged predator, tadpole density and trematode exposure on tadpole development, survival and activity.

TABLE B1. Analysis of variance for effects of a caged predator, tadpole density and trematode exposure on tadpole development, survival and activity in the Exposure experiment. Significant P-values are in bold text. All models had 21 error degrees of freedom.

		Larval period		**Mass**		**Survival**		**Activity**	
ANOVA	**df**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
Block	3	< 0.1	0.968	1.1	0.372	0.7	0.54	1.2	0.333
Predator	1	17.5	< **0.001**	0.9	0.365	1	0.321	23.5	< **0.001**
Density	1	80.9	< **0.001**	51.1	< **0.001**	12.1	**0.002**	7.3	**0.013**
Infection	1	5.2	**0.033**	< 0.1	0.932	8.6	**0.008**	10.1	**0.004**
Predator * Density	1	0.5	0.489	< 0.1	0.944	0.4	0.528	5.8	**0.026**
Predator * Infection	1	2.1	0.158	0.3	0.604	0.2	0.651	< 0.1	0.859
Density * Infection	1	1.3	0.265	0.9	0.35	1.6	0.224	6.8	**0.017**
Predator * Density * Infection	1	0.4	0.557	0.3	0.575	< 0.1	0.874	0.7	0.399

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Appendix C. Graphs showing effects of density and delayed tadpole development on susceptibility to *Echinostoma trivolvis* infection.

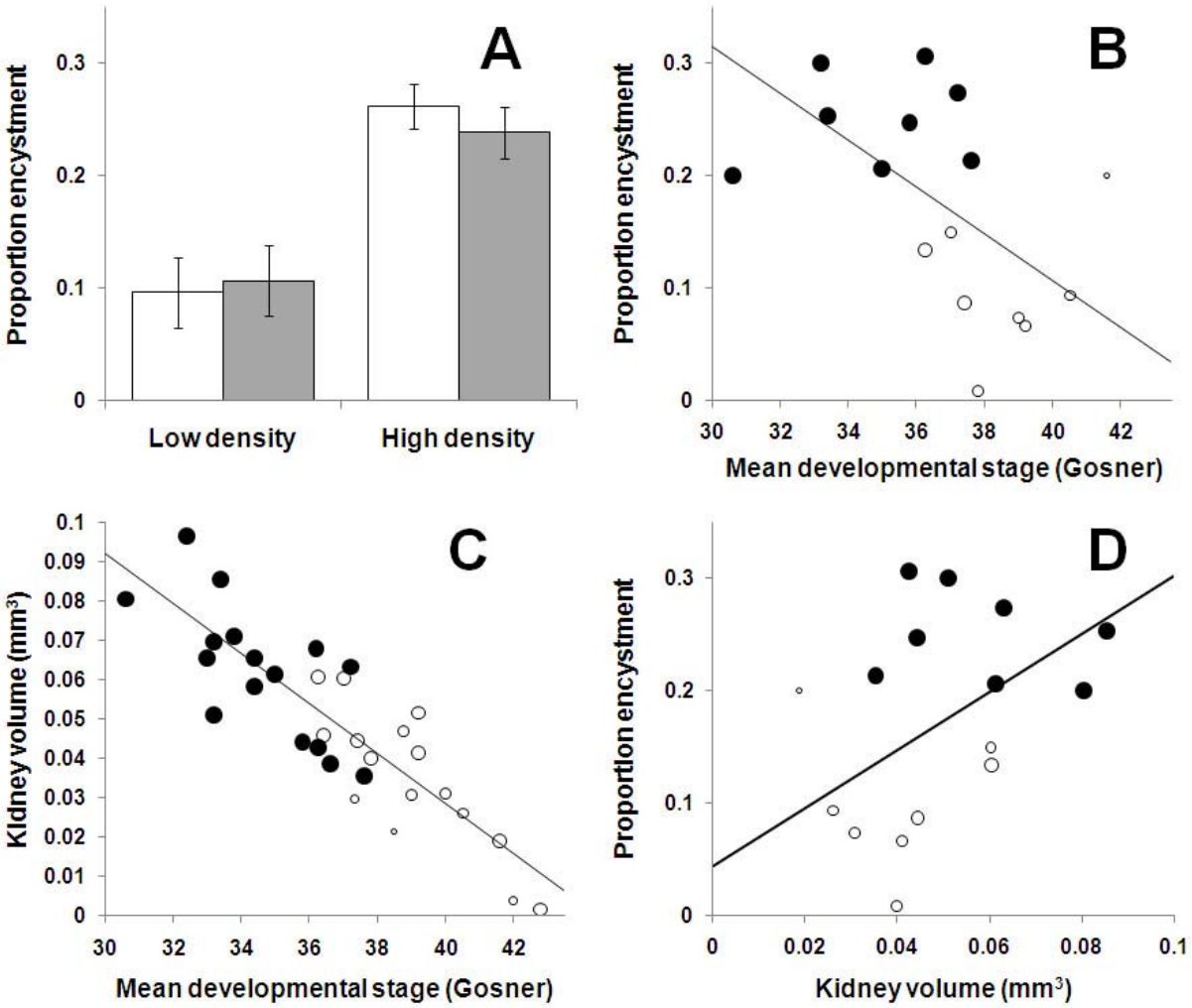

FIG. C1. Effects of delayed tadpole development on susceptibility to *Echinostoma trivolvis* infection. (A) The effect of density (15 vs. 30 tadpoles) on proportion encystment could be partly explained by: (B) the effect of tadpole developmental stage (Gosner 1960) on proportion encystment, and (C) the effect of development on kidney (pronephros) volume. (D) The relationship between kidney volume and proportion encystment. Filled circles indicate high density mesocosms, and circle sizes indicate statistical weights (B, D: product of sample sizes for staged tadpoles and experimentally infected tadpoles from each mesocosm; C: sample size for staged tadpoles, max = 5). Gray bars in (A) indicate the presence of a caged predator. Error bars = SE.

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Appendix D. Statistics and graphs describing effects of density, predation and development on leukocyte parameters.

Effects of density, predation and development on leukocyte parameters

There was no multivariate effect of the caged newt predator on tadpole leukocytes in the susceptibility experiment (Wilk's $\lambda = 0.891$, $F_{5,21} = 0.5$, $P = 0.781$). There was a significant multivariate effect of density ($\lambda = 0.588$, $F_{5,21} = 2.8$, $P = 0.045$), but none of the individual immune cells had strong enough effects in a direction that could account for increased susceptibility to *E. trivolvis* infection at high tadpole density. In univariate analyses, tadpoles at high density had significantly more basophils ($F_{1,26} = 5.5$, $P = 0.027$) and trends toward more lymphocytes ($F_{1,26} = 4.1$, $P = 0.053$) and fewer neutrophils ($F_{1,26} = 3.4$, $P = 0.076$), but there were no significant effects on eosinophils or monocytes (both $P > 0.3$). Basophils are thought to be an important component of the immune response to helminths (Min and Paul 2008), so higher levels are unlikely to account for decreased resistance to *E. trivolvis* infection. High density tanks also had a significantly lower neutrophil-to-lymphocyte ratio than low density tanks ($F_{1,26} = 7.7$, $P = 0.010$), opposite the predicted effect of physiological stress associated with crowding (Davis *et al.* 2008). One control mesocosm was excluded from the leukocyte analyses because there were no usable blood smears.

There was also a significant multivariate effect of developmental stage on leukocytes when tested in the absence of other variables ($\lambda = 0.622$, $F_{5,25} = 3.0$, $P = 0.028$), and this effect correlated with the density effect so that neither was significant when both were included in the model (both $P > 0.3$). The univariate effects of development on leukocyte counts paralleled the density effects, with basophils decreasing ($F_{1,29} = 12.8$, $P = 0.001$) and the neutrophil-to-lymphocyte ratio increasing ($F_{1,29} = 5.0$, $P = 0.033$) with development (Fig. D1). Neither developmental stage nor density remained significant in any univariate model including the other predictor (all $P > 0.1$), except for basophils, for which developmental stage remained a significant predictor ($F_{1,28} = 6.2$, $P = 0.020$) but density did not ($P = 0.946$). These results suggest that the observed effects of tadpole density on leukocytes were caused by delayed development coupled with developmental changes in blood leukocyte levels.

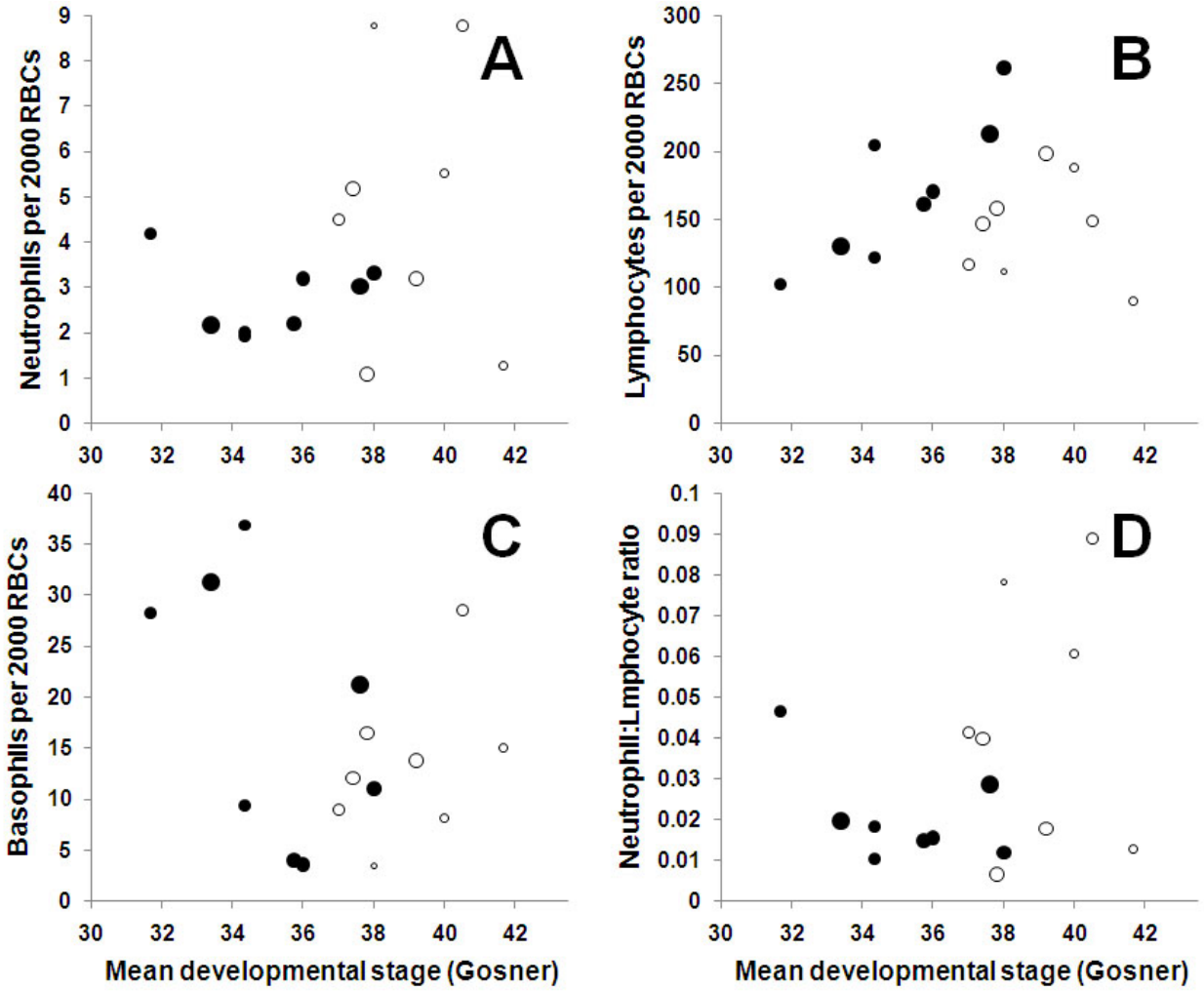

FIG. D1. Relationships between tadpole developmental stage (Gosner 1960) and blood parameters, including (A) neutrophils, (B) lymphocytes, (C) basophils, and (D) the neutrophil-to-lymphocyte ratio. Filled circles indicate high density mesocosms and circle sizes indicate weights (sample size of blood smears, max = 5).

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